DNA G-quadruplex and i-motif structure formation is interdependent in human cells.

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Supporting Information Placeholder

ABSTRACT: Guanine and cytosine-rich nucleic acid sequences have the potential to form secondary structures such as G-quadruplexes and i-motifs, respectively. We show that stabilisation of G-quadruplexes using small molecules destabilises the i-motifs, and vice versa, indicating these gene regulatory controllers are interdependent in human cells. This has important implications as these structures are predominately considered as isolated structural targets for therapy, but their interdependency highlights the interplay of both structures as an important gene regulatory switch.

Dynamic structural transitions between the common B-DNA and alternative DNA conformations provides an additional layer of regulatory control in gene expression.[1–4] G-quadruplex (G4) and i-motif (iM) are two important

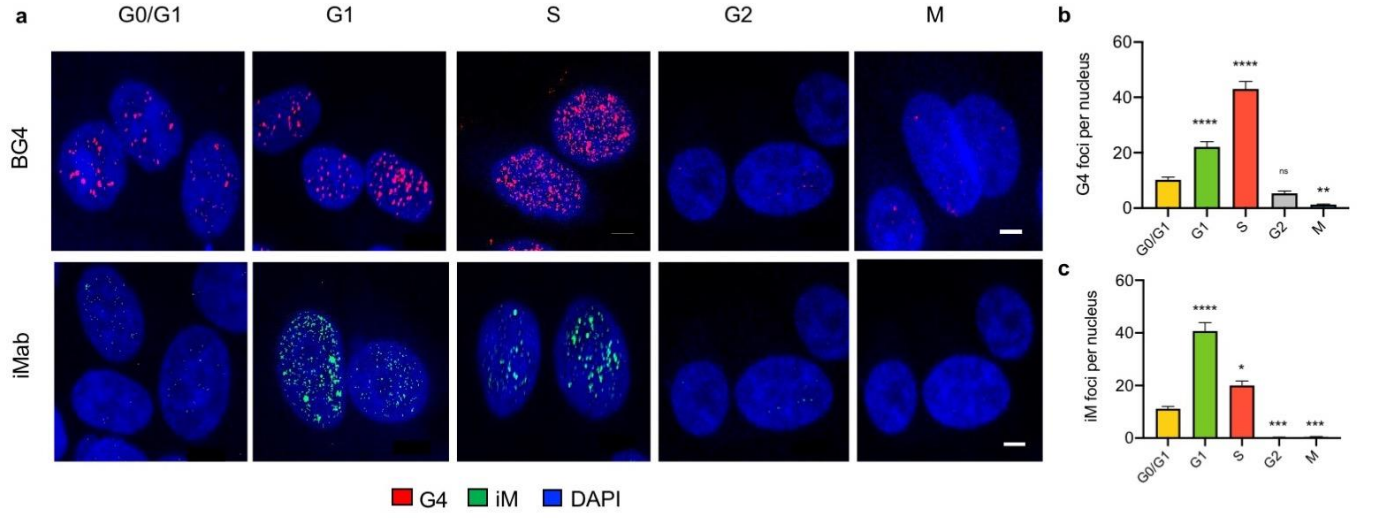

Figure 1. Visualisation and quantification of G4 and iM structures throughout the MCF-7 cell cycle. (a) Representative images of G4 foci (red) and iM foci (green) in MCF-7 nuclei (blue) at each cell cycle stage. Image scale bar, 5 μm. **(b)** Quantification of G4 foci throughout the cell cycle. **(c)** Quantification of iM foci throughout the cell cycle. n = 60 to 107 nuclei, and mean and S E.M. were calculated from two biological replicates. Statistical significance was assessed by one-way ANOVA and Tukey's multiple comparisons test. Significance shown is relative to G0/G1. ***p*<0.01, ****p*<0.001, *****p*<0.0001.

classes of non-canonical DNA structures that form within certain guanine- and cytosine-rich regions of the human genome, respectively. As iM structures are formed via a stack of intercalating hemiprotonated cytosine base pairs (C+:C), it was initially thought that iM formation required slightly acidic pH, however, it has now been established that these structures form at physiological pH within cellular environments.[5,6] G4 structures are formed from pi-stacked planar G-tetrads, where each G-tetrad consists of four guanine bases held together by Hoogsteen hydrogen bonding, and are further stabilised by physiologically relevant cations.[7–10] G4 and iM folding mechanisms have been used to predict the propensity of their formation across the genome and their overrepresentation in regulatory regions.[5,11] Furthermore, their structural features have been widely exploited as scaffolds for the design of small molecule targeting ligands to stabilise G4 and iM structures and thereby regulate gene expression.[12–16] Most G4s identified to date have been classified as transcriptional suppressors, in contrast, there is increasing evidence that iM structures may play the opposite role in gene regulation in activating gene expression.[1,3,13] Given that these G- and C-rich sequences occur in complimentary strands of duplex DNA, understanding G4 and iM dynamics relative to each other is crucial for developing targeted therapies. Previous in vitro studies of duplex DNA containing complementary G- and C-rich strands have reported that G4 and iM formation are mutually exclusive.[12,13,17] Here, we demonstrate for the first time that inducing and stabilising G4 or iM destabilises the other in human cells, potentially perturbing dynamic structural transitions across the genome. In particular, we show that targeting one of these non-canonical DNA structures using small molecule stabilising ligands induces destabilisation of the other. Given that these non-canonical structures are important genomic regulatory elements, their dynamic interdependence suggests that it is not only active stabilisation but also concomitant destabilisation of the other that governs gene regulation.

During transcription local unwinding of duplex DNA facilitated by negative supercoiling stress generates transiently exposed single-strand segments enabling G4 and iM structures to form.[18] In order to establish the propensity of formation of G4 and iM secondary structures in human cells, it was pivotal to comprehensively map G4 and iM formation throughout the entirety of the cell cycle. This is essential to map both the prevalence of these secondary structures and the biological roles they play in regulating gene expression. Using recent breakthroughs in the development of structure-specific antibodies BG4[10] and iMab[6], to detect G4 and iM structures, respectively, in human cells, we quantified their formation at the five main cell cycle stages (G0/G1, G1, S, G2, and M) in the MCF-7 cell line (cell cycle arrest was confirmed by flow cytometry, Supplementary Fig. 2). We confirmed the propensity for G4 formation was maximal at S phase, during DNA replication, and iM formation was highest at G1 during high levels of transcription (Fig. 1).[6,10] Not surprisingly, G4 and iM formation were lowest during G2 and M phase (Fig. 1), when cellular process such as transcription and replication are restricted, and histone modifications lead to chromatin condensation.[19] This suggests that the

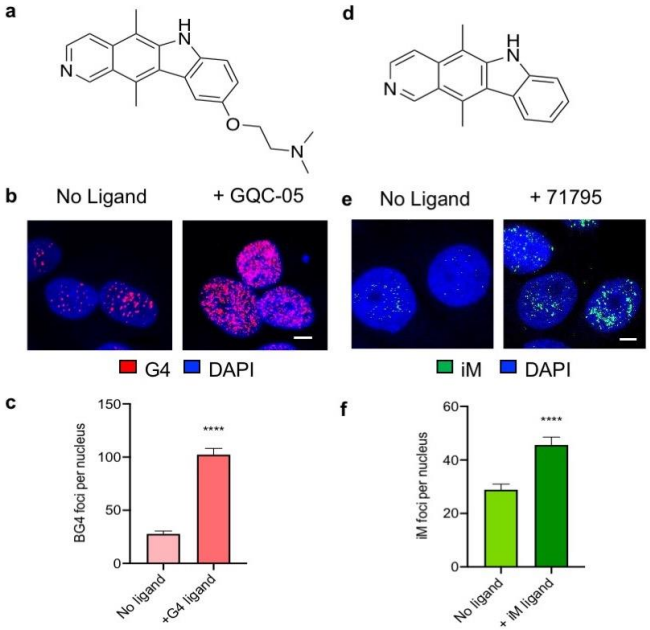

Figure 2. Stabilisation of G4 and iM structures within MCF-7 nuclei. (a) Chemical structure of GQC-05 (NSC338258). **(b)** Representative images of G4 foci in MCF-7 nuclei before and after treatment with GQC-05 (10 μM). **(c)** Stabilisation of G4s by GQC-05 increases G4 foci count. **(d)** Chemical structure of 71795 (NSC71795). **(e)** Representative images of iM foci in MCF-7 nuclei before and after treatment with 71795 (10 μM). **(f)** Stabilisation of iMs by 71795 increases iM foci count. Image scale bar, 5 μm. **(c,f)** Quantification of G4 and iM foci in MCF-7 nuclei, n= 138-213. Mean and S.E.M. calculated from two biological replicates. Statistical significance was assessed by t-test, ****p>0.0001.

number of these secondary structures formed within the genome coincides with chromatin accessibility.[11] Furthermore we validated that mimosine, RO-3306, and colcemid, which were used to arrest the cell cycle, did not affect the stabilisation of G4 or iM structures, confirming that the changes in the number of detectable G4 and iM were indeed related to cell cycle stage (Supplementary Fig. 3).

In order to establish whether treatment of cells with small molecules designed to stabilise G4 and iM would induce a concomitant increase in the number of detectable structurers, we treated unsynchronised MCF-7 cells with ellipticine analogues, either a G4 stabilising ligand (GQC-05, 10 μM) or iM stabilising ligand (71795, 10 μM) for 1 hr (Fig. 2). The efficacy of GQC-05 to selectively stabilise G4 DNA has been previously reported.[20]

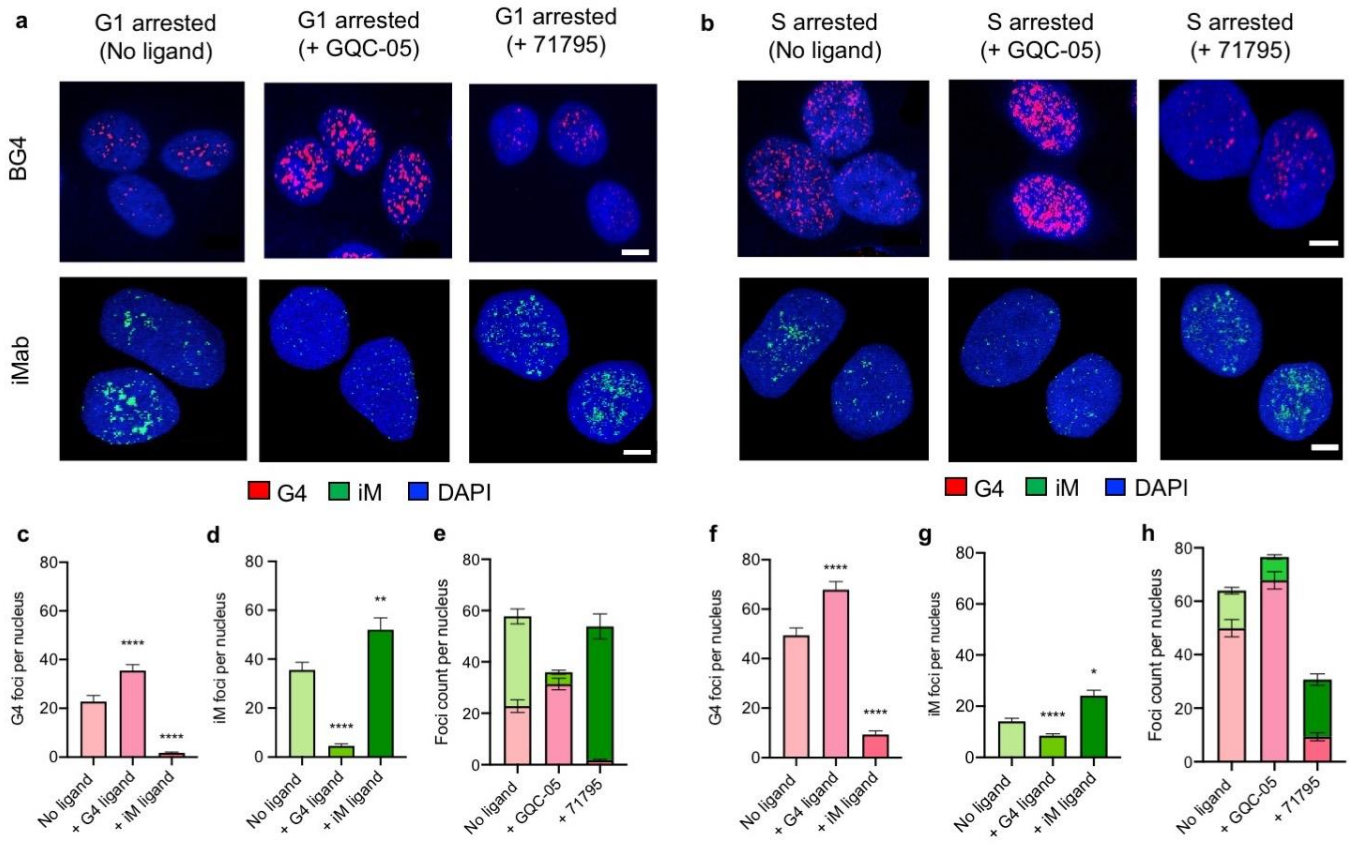

Figure 3. G4 and iM formation is interdependent in human cell nuclei. (a) Immunofluorescence of G4 (red) and iM (green) foci in MCF-7 nuclei (blue) at G1 in the presence of GQC-05 (10 μM) and 71795 (10 μM). **(b)** Immunofluorescence of G4 and iM in S phase arrested MCF-7 nuclei, after treatment with GQC-05 (10 μM) and 71795 (10 μM). **(c-h)** Quantification of G4 and iM foci in G1 **(c-e)** and S **(f-h)** arrested MCF-7 cells. Sample sizes ranged from n= 168 to 204 nuclei from two biological replicates. Statistical significance is shown relative to the no ligand control and was assessed by one-way ANOVA and Tukey's multiple comparisons test. *p<0.1, **p<0.01, ***p<0.001, ****p<0.0001. **(e,h)** Stack plots showing the relative abundance of G4 and iM foci in G1 **(e)** and S **(h)** arrested MCF-7 nuclei.

In the case of iM ligand (71795) we performed biophysical analysis to demonstrate high preference for iM DNA structures over G4 and duplex DNA (Supplementary Table 2). Treatment of MCF-7 cells with GQC-05 and 71795 at 10 μM resulted in statistically significant (p < 0.0001) increases in the number of nuclear G4 and iM foci respectively (Fig. 2) confirming ligand-induced stabilisation within the cellular environment. Given that both these structural transitions are dynamic, small molecule binding stabilises the structures, locking them into position.

We next examined the consequences of stabilising one structure on the prevalence of the other. Given that G4 formation is maximal at early S phase, and iM formation is maximal at G1, we synchronised MCF-7 cells at these two stages, followed by treatment with GQC-05 (10 μM, 1 hr) and 71795 (10 μM, 1 hr). In the case of cells arrested in G1 stage, treatment with GQC-05 resulted in a significant increase in G4 and a decrease in iM formation (Fig. 3c,d,g). Treatment with 71795 resulted in an increase in iM and significant decrease in the G4 formation (Fig. 3c,d,g). Similar results were obtained for cells arrested in S phase (Fig. 3e,f,h). This result suggests that during G1 phase, when there is high propensity towards the formation of iM, the stabilisation of G4 structures occurs at the expense of the number of iM structures. Similarly, during S phase, when there is high propensity towards the formation of G4, treatment with the iM-stabilising ligands triggers the unfolding of G4 structures. This indicates that G4 and iMs are interdependent and act as structural controllers of each other's formation.[12,13] We also treated unsynchronized cells with GQC-05 and 71795 sequentially, 71795 and GQC-05 sequentially and with both ligands simultaneously, and found that these ligands act competitively (Fig. 4).

In conclusion our findings confirm that while G4 and iM formation in human cells is dependent on chromatin accessibility and cell cycle progression, they are also highly dependent on the formation of each other. In particular we show that stabilisation of G4 structures at G1 phase (when iMs are most prevalent) causes a reduction in the number of iM structures, while stabilising iMs at S

phase (when G4s are most abundant) results in a decrease in the number of observed G4 structures. Our results provide insights into the nature of G4 and iM formation and offer a basis for future biological studies and therapeutic targeting for diseases.

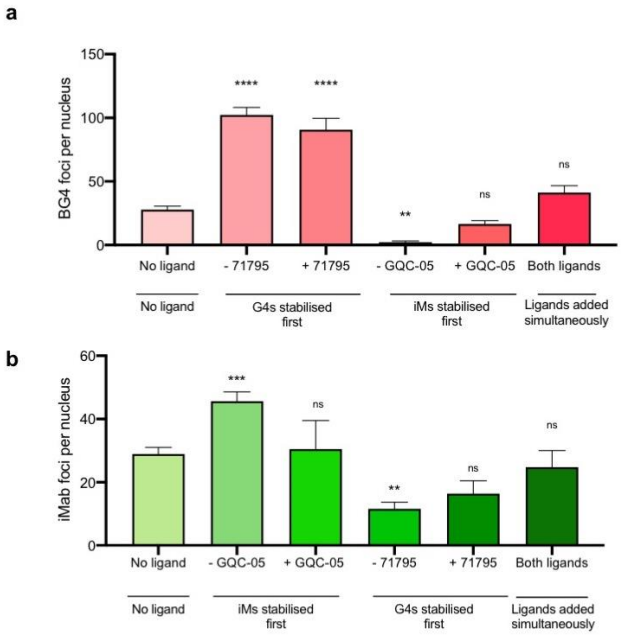

Figure 4. BG4 and iMab quantification in MCF-7 cell nuclei with G4 and iM stabilization. GQC-05 and 71795 ligands were used to stabilise G4 and iM structures, respectively. MCF-7 cell nuclei were treated with GQC-05 (10 μM) after iM stabilization and as well as treatment with 71795 (10 μM) after G4 stabilization. GQC-05 (10 μM) and 71795 (10 μM) were also added simultaneously. **(a)** BG4 quantification in MCF-7 nuclei. **(b)** iMab quantification in MCF-7 nuclei. Mean and S.E.M. are displayed. Sample sizes ranged from n= 47 to 95 nuclei from three technical triplicates. Statistical significance relative to the no ligand control is displayed. ns = non-significant, *p<0.1, **p<0.01, ***p<0.001, ****p<0.0001.

ASSOCIATED CONTENT

This information includes flow cytometry graphs, statistical significance results, immunofluorescence experiments, and FRET spectra. This information is available free of charge via the Internet at http://pubs.acs.org.

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